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学 位 論 文

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Teneligliptin decreases the uric acids levels by reducing xanthine dehydrogenase expression in white adipose tissue of the male Wistar rats.

(テネリグリプチンはラット白色細胞においてキサンチンデヒドロゲナーゼの発現を抑制し尿酸値を低下させる)

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代謝制御学分野

腎臓高血圧・糖尿病内分泌学講座

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学位論文

**Teneligliptin decreases the uric acids levels by reducing xanthine dehydrogenase
expression in white adipose tissue of the male Wistar rats.**

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Abstract

Purpose: Uric acid is one of the risk factors for cardiovascular diseases. Recently, it has been reported that adipose tissue produces and secretes uric acid through xanthine oxidoreductase (XOR), and that its production is enhanced in obesity. In this study, the effects of teneligliptin on uric acid metabolism were examined in male Wistar rats.

Methods: The male Wistar rats were fed a normal chow diet (NCD) or a 60% high-fat diet (HFD) with or without teneligliptin (~ 4.0 mg/kg/day) for 4 weeks.

Results: Body weight was not significantly different between the control and teneligliptin groups, while body weight of the HFD-fed rats was significantly greater than in the NCD-fed rats. The plasma uric acid level was not significantly different between the control and teneligliptin groups under the NCD condition. However, the plasma uric acid level was significantly decreased (by 21%) in the HFD-fed teneligliptin treated rats compared to the HFD-fed control rats. To investigate the molecular mechanisms of this effect of teneligliptin, quantitative real-time PCR analysis was performed. The expression levels of xanthine dehydrogenase (Xdh), the major form of XOR in tissues, in liver and epididymal adipose tissue of NCD-fed rats were not altered by teneligliptin treatment. On the other hand, Xdh expression was reduced significantly by 32% in the epididymal adipose tissue of the HFD-fed teneligliptin treated rats compared that of HFD-fed control rats, whereas Xdh expression in liver did not change significantly in either group. Furthermore, teneligliptin significantly decreased Xdh expression in 3T3-L1 adipocytes. DPP-4 treatment significantly increased Xdh expression by 49% in 3T3-L1 adipocytes. With DPP-4 pretreatment, teneligliptin significantly decreased Xdh mRNA expression by 26% compared to the DPP-4-treated 3T3-L1 adipocytes.

Conclusions: These data suggest that teneligliptin reduces uric acid levels by suppressing Xdh expression in epididymal adipose tissue of obese subjects.

Key Words: Teneligliptin, uric acids, xanthine dehydrogenase, adipose tissue

Introduction

The prevalence of type 2 diabetes mellitus has increased dramatically worldwide, mainly because of changes in lifestyle, such as decreasing exercise and increasing high-fat diets. Obesity is the hallmark of the metabolic syndrome and represents a major global health problem that is frequently associated with the development of chronic diseases, including type 2 diabetes mellitus and cardiovascular disease (1). A complex inter-organ cross-talk scenario between adipose tissue and other central and peripheral organs underlies the progression of these diseases, with adipose tissue on top of the cross-talk hierarchy (2).

Use of dipeptidyl peptidase-4 (DPP-4) inhibitors is a strategy for glucose-lowering treatment in type 2 diabetic patients (3). DPP-4 inhibitors were first approved for clinical use in 2006 with the DPP-4 inhibitor sitagliptin, and, thereafter, many other DPP-4 inhibitors have been introduced into clinical practice (4). Teneligliptin, one of the DPP-4 inhibitors, has a unique structure characterized by five consecutive rings, which produce a potent and long-lasting effect (5,6). The gut-derived glucagon-like peptide-1 (GLP-1) plays important roles in both postprandial and long-term glucose homeostasis by increasing glucose-stimulated insulin secretion and inhibiting glucagon secretion (7). DPP-4 is an enzyme that rapidly degrades circulating GLP-1, and, therefore, DPP-4 inhibitors prevent the inactivation of GLP-1 and, consequently, increase the circulating active GLP-1 levels above physiological levels that have antidiabetic actions (3). In addition, DPP-4 is a ubiquitously expressed transmembrane glycoprotein that cleaves N-terminal dipeptides from a variety of substrates, including growth factors and hormones, neuropeptides, and chemokines, such as incretin hormones (8). The expression of DPP-4 is substantially dysregulated in a variety of disease states, including inflammation, cancer, obesity, and diabetes (9). It has also been reported that DPP-4 released

from adipose tissue is positively correlated with an increasing risk score for the metabolic syndrome. DPP-4 release is strongly correlated with adipocyte size, potentially representing an important source of DPP-4 in obesity. Therefore, it has been suggested that DPP-4 may be involved in linking adipose tissue and the metabolic syndrome (10).

Recently, it has been reported that adipose tissue produces and secretes uric acid through xanthine oxidoreductase (XOR), and that its production is enhanced in obesity (11). Uric acid is also one of the risk factors for cardiovascular diseases (11,12). Xanthine oxidase (XO), which is a variant of XOR, induces oxidative stress in the process of uric acid production. On the other hand, cardiac insufficiency and obesity produce a hypoxic state that leads to oxidative stress, which activates XO. Oxidative stress is highly relevant to aging and the development of various aging-related cardiovascular diseases and insulin resistance. Thus, inhibition of XO suppresses the oxidative stress of uric acid, which improves vascular endothelial dysfunction, heart failure, and insulin resistance (12).

We hypothesized that teneligliptin might have pleotropic effects in these tissues. DPP-4 inhibitors can improve glycemic control by prolonging the effect of GLP-1. Many studies have already reported that teneligliptin improves not only blood glucose, but also the lipid profile and early-phase insulin secretion (13-17).

In the current study, the effect of teneligliptin on uric acid metabolism was examined in male Wistar rats. It was found that teneligliptin decreased uric acid levels in high-fat diet (HFD)-fed rats, but not normal chow diet (NCD)-fed rats.

Methods

Materials

Teneligliptin was donated by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). 3T3-L1 preadipocytes were purchased from American Type Cell Collection (Manassas, VA, USA). Male Wistar rats were obtained from Charles River Laboratory Japan, Inc. (Kanagawa, Japan). The high-fat diet (HFD) (60% w/w, #D12492) was purchased from Research Diet Inc. (New Brunswick, NJ, USA). Isoflurane was purchased from Intervet K.K (Tokyo, Japan). Pentobarbital was purchased from Kyoritsu Pharmaceutical Co. (Tokyo, Japan). DMEM, streptomycin, trypsin, fetal bovine serum (FBS), and TRIzol reagent were from Invitrogen Life Technologies (Carlsbad, CA, USA). The RNeasy kit was obtained from QIAGEN Inc. (Valencia, CA, USA). The iScript cDNA Synthesis Kit and the iQ SYBR Green Supermix were from Bio-Rad Laboratories (Richmond, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

Animal studies

Six-week-old male Wistar rats (Charles River Laboratory Japan, Inc) were housed individually under controlled 12-hour light, 12-hour dark cycles and temperature conditions (25°C) and had free access to water and a normal chow diet (NCD) or 60% HFD (Research Diet, Inc). The male Wistar rats were fed NCD or HFD with or without teneligliptin (~4.0 mg/kg body weight/day) for 4 weeks. The rats received a fresh diet every 3 days, and food consumption rates and body weight gains were monitored every 3 days. After the indicated diet for 4 weeks, the rats were fasted for 6 hours and then anesthetized in an induction chamber with 3% isoflurane. Plasma samples were obtained in the presence of EDTA-Na

from the aorta of rats, and they were then promptly euthanized with pentobarbital (180 mg/kg body weight). The epididymal adipose tissue and liver samples were dissected and immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the NIH and were approved by the Animal Subjects Committee of Fukushima Medical University, Japan.

Uric acid level measurement

Plasma uric acid levels were analyzed by a private laboratory (SRL Laboratory, Tokyo, Japan).

Cell culture and cell treatment

Mouse 3T3-L1 cells were cultured and differentiated as described previously (18,19). Unless otherwise indicated, adipocytes were used 14 days after differentiation. After incubation in serum-free DMEM high glucose for 3 hours, differentiated 3T3-L1 adipocytes were treated with the indicated concentration of teneligliptin, and/or 200 ng/mL DPP-4 for 1, 3, 6, or 12 hours before each assay.

Quantitative real-time reverse-transcription PCR analysis

Total RNA samples were extracted from cells, epididymal adipose tissue, and liver samples with TRIzol reagent, and total RNA was further purified using the RNeasy kit with RNase-free DNase I treatment according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's

instructions (Bio-Rad Laboratories, Inc). Quantitative real-time PCR was performed with a Bio-Rad system using iQ SYBR Green Supermix and specific primer pairs (Table 1) selected with Primer Express software (Applied Biosystems). The relative mass of specific RNAs was calculated by the comparative cycle of threshold detection method according to the manufacturer's instructions.

Statistical analysis

Data are presented as means \pm SEM. Statistical significance was tested with repeated measures analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$.

Results

Overall animal characteristics

The effect of teneligliptin was examined in both NCD-fed and HFD-fed male Wistar rats. Table 2 shows some of the general characteristics of the teneligliptin and control groups at 4 weeks. In the NCD-fed rats, body weight, average daily food consumption, and fasting plasma glucose levels were not significantly different between the teneligliptin and the control groups. Compared with the control group, HFD feeding led to a significant increase in body weight, but no significant change in fasting plasma glucose levels. In the HFD-fed rats, body weight, average daily food consumption, and fasting plasma glucose levels were also not significantly different between the two groups.

Effects of teneligliptin on uric acid metabolism in male Wistar rats

In the NCD-fed rats, plasma uric acids levels were not significantly different between the teneligliptin and the control groups. Interestingly, in the HFD-fed rats, plasma uric acid levels were significantly decreased by 21% (from 0.34 ± 0.02 mg/dL to 0.27 ± 0.02 mg/dL; $P < 0.05$) in the teneligliptin group compared with the control group.

Effect of teneligliptin on the uric acid synthesis gene (xanthine dehydrogenase (Xdh)) in liver and epididymal adipose tissues of male Wistar rats

To explore the potential cellular mechanisms involved in the teneligliptin-induced decrease of plasma uric acid levels, quantitative real-time PCR analysis was performed on total RNA from epididymal adipose tissue and a liver sample in selected chow-fed rats. The expression levels of xanthine dehydrogenase (Xdh), which is one of the key enzymes in the

uric acid synthesis pathway, were measured. The expression of Xdh in liver tissue was not significantly altered by teneligliptin treatment under either NCD or HFD conditions (Figure 2A). On the other hand, the expression of Xdh in epididymal adipose tissue was reduced significantly by 32% ($P < 0.01$) in the HFD-fed teneligliptin treated rats compared to the HFD-fed control rats, whereas the expression of Xdh in epididymal adipose tissue did not change significantly in the NCD-fed control rats and the NCD-fed teneligliptin treated rats.

Effects of teneligliptin on uric acid synthesis gene (Xdh) in 3T3-L1 adipocytes

Next, whether treatment with teneligliptin altered the expression of Xdh in 3T3-L1 adipocytes was investigated. Quantitative real-time RT-PCR was performed on total RNA from serum-starved 3T3-L1 adipocytes that were treated with or without teneligliptin (1, 5, 10 μ M) for 3 hours. As shown in Figure 3, teneligliptin significantly decreased the expression of Xdh by 45% ($P < 0.01$), 35% ($P < 0.01$), and 34% ($P < 0.01$) at 1, 5, and 10 μ M concentrations, respectively, in 3T3-L1 adipocytes. Moreover, as shown in Figure 4, treatment for 12 hours with DPP-4 (200 ng/mL), a novel adipocytokine that impairs insulin sensitivity in an autocrine and paracrine fashion (10), significantly increased the expression of Xdh by 49% ($P < 0.01$), whereas DPP-4 treatment for at least 6 hours did not significantly change the expression of Xdh. With DPP-4 (200 ng/ml) pretreatment for 12 hours, teneligliptin significantly decreased the expression of Xdh by 19% ($P < 0.05$), 30% ($P < 0.05$), and 26% ($P < 0.01$) at 1, 5, and 10 μ M concentrations, respectively, compared to the DPP-4-treated 3T3-L1 adipocytes (Figure 5).

Discussion

Teneligliptin, a novel, highly selective DPP-4 inhibitor, is an antidiabetic drug that improves glycemic control without causing weight gain or increasing hypoglycemic risk in patients with type 2 diabetes mellitus. Although the glycemic efficacy of teneligliptin is well-known, the non-glycemic efficacy and mechanisms of teneligliptin are not well understood. In this study, under the HFD condition, but not under the NCD condition, teneligliptin decreased plasma uric acid levels by reducing Xdh expression in adipose tissue but not liver. In addition, in 3T3-L1 adipocytes, DPP-4 increased Xdh expression, and teneligliptin decreased DPP-4-induced Xdh expression. As shown in Figure 6, these findings raised the possibility that teneligliptin may decrease plasma uric acid levels by inhibiting DPP4 activity in adipose tissue.

DPP-4 is ubiquitously expressed on numerous different cell types, among which are epithelial cells, fibroblasts, and leukocyte subsets. Mechanisms that regulate DPP-4 gene transcription and enzymatic activity are not fully understood. It has recently been reported that adipocytes released DPP-4 in a differentiation-dependent manner (10). In addition, DPP-4 expression in adipose tissue was increased in obese compared to lean subjects, a fact that is reflected by the increased release of DPP-4 from adipose tissue explants of obese subjects compared with lean subjects (10). Circulating DPP-4 concentrations were increased in obese subjects and correlated with parameters of the metabolic syndrome, such as body mass index, waist circumference, and plasma fasting insulin concentration (10). Furthermore, DPP-4 exerted autocrine and paracrine effects and impaired insulin signaling (10). Thus, DPP-4 is a novel adipocytokine and biomarker, and it is a potential link between obesity and the metabolic syndrome.

A central finding in the present study is that teneligliptin, one of the DPP-4 inhibitors, reduced plasma uric acid levels in HFD-fed rats by downregulation of Xdh expression in adipose tissue. This observation is strongly associated with the upregulation of DPP-4 expression and release in adipose tissue of obese subjects. Therefore, DPP-4 increases Xdh expression and teneligliptin decreases DPP-4-induced Xdh expression in 3T3-L1 adipocytes. DPP4 stimulates XDH, and then XDH promotes the production of uric acid.

Hyperuricemia is a key risk factor for the development of gout, renal dysfunction, hypertension, dyslipidemia, diabetes, and obesity. Hyperuricemia occurs as a result of the increased uric acid production, impaired renal uric acid excretion, or a combination of the two (20). Endogenous production of uric acid is mainly from the liver, intestines, and other tissues such as muscles, kidney, and vascular endothelium (21). Recently, it has been reported that adipose tissue produces and secretes uric acid through XOR, and that its production is enhanced in obesity (11). In mammals, XOR can exist in two enzymatic forms: XDH and XO. The present results suggest that inhibition of Xdh expression in adipose tissue is important in the treatment of diabetes. Another study also suggested that knockdown of XOR promoted transcription of a PPAR γ (22). Oxidative stress associated with XO production through the process of uric acid damages fat cells and vascular endothelial cells. In addition, in 3T3-L1 adipocytes, uric acid increased monocyte chemotactic protein (MCP-1) gene expression, which induced macrophages and inflammation, and decreased the expression of adiponectin. In addition, adding an antioxidant agent suppress this reaction (23). Aggravation of insulin resistance by such cell inflammation would exacerbate sugar metabolism itself. In other words, hyperuricemia can be related to multiple risk factors for atherosclerosis caused by the fat

tissue. To suppress the uric acid level is very important in diabetes therapy, because the goal of diabetes treatment may be to reduce the complications.

Many other DPP-4 inhibitors have been introduced into clinical practice (4). It has been reported that sitagliptin increases the serum uric acid level (24). In addition, another study reported that switching from sitagliptin to vildagliptin decreased the serum uric acid level (25). On the other hand, it has been reported that linagliptin decreased the serum uric acid level by suppressing xanthine oxidase activity (26). Although the precise mechanism underlying these differences by the kind of DPP-4 inhibitor is unknown, one possibility exists, which likely arises from differential transferability to adipose tissue. Furthermore, in the present study, it was 12 hours later that the Xdh expression increased to its highest level after pretreatment with DPP-4 in 3T-3L1 adipocytes. Therefore, it is also important to maintain plasma concentrations of DPP-4 inhibitors. Tenueligliptin can maintain plasma concentrations because of its terminal elimination half-life of 26.9 hours (5).

In conclusion, the present data suggest that tenueligliptin reduces uric acid levels by suppressing Xdh expression in epididymal adipose tissue of obese subjects. Therefore, tenueligliptin is more effective for controlling blood glucose and hyperuricemia in patients with type 2 diabetes mellitus.

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Figure Legends

Figure 1. Effects of teneligliptin on plasma uric acid levels in male Wistar rats

The rats were fed NCD or HFD with or without teneligliptin (~4.0 mg/kg body weight/day) for 4 weeks. After 4 weeks, the rats were fasted for 6 hours, and then plasma samples were obtained in the presence of EDTA-Na from the aorta of rats under anesthesia. The plasma uric acid levels were measured by a private laboratory (open squares, control group, NCD-fed rats: n = 10, HFD-fed rats: n = 9; closed squares, teneligliptin group, NCD-fed rats: n = 9, HFD-fed rats: n = 9). Data are the means \pm SEM. *, $P < 0.05$ vs the control rats.

Figure 2. Effects of teneligliptin on xanthine dehydrogenase (Xdh) in liver (A) and epididymal adipose tissues (B) of male Wistar rats

Total RNAs extracted from liver tissue and epididymal adipose tissue of the control group (open squares: NCD-fed rats: n = 10, HFD-fed rats: n = 9) and the teneligliptin group (closed squares: NCD-fed rats: n = 9, HFD-fed rats: n = 9) were used for gene expression analysis of Xdh. Levels of Cph were used for normalization of sample loading. Data are the means \pm SEM. Data are expressed relative to NCD-fed control values. **, $P < 0.01$ vs control rats.

Figure 3. Effects of teneligliptin on Xdh in 3T3-L1 adipocytes

Serum-starved 3T3-L1 adipocytes were treated without (open squares) or with teneligliptin (closed squares 1, 5, 10 μ M) for 3 hours. Total RNAs extracted from all cells were used for gene expression analysis of Xdh. Levels of Cph were used for normalization of

sample loading. Data are the means \pm SEM of 3 independent experiments (1 experiment performed with 6 samples). Data are expressed relative to control values. **, $P < 0.01$ vs control cells.

Figure 4. Effects of DPP-4 on Xdh in 3T3-L1 adipocytes

Serum-starved 3T3-L1 adipocytes were treated without (open squares) or with DPP-4 (closed squares 200 ng/mL) for the indicated periods (1, 3, 6, 12 hours). Total RNAs extracted from all cells were used for gene expression analysis of Xdh. Levels of Cph were used for normalization of sample loading. Data are the means \pm SEM of 3 independent experiments (1 experiment performed with 6 samples). Data are expressed relative to control values. **, $P < 0.01$ vs control cells.

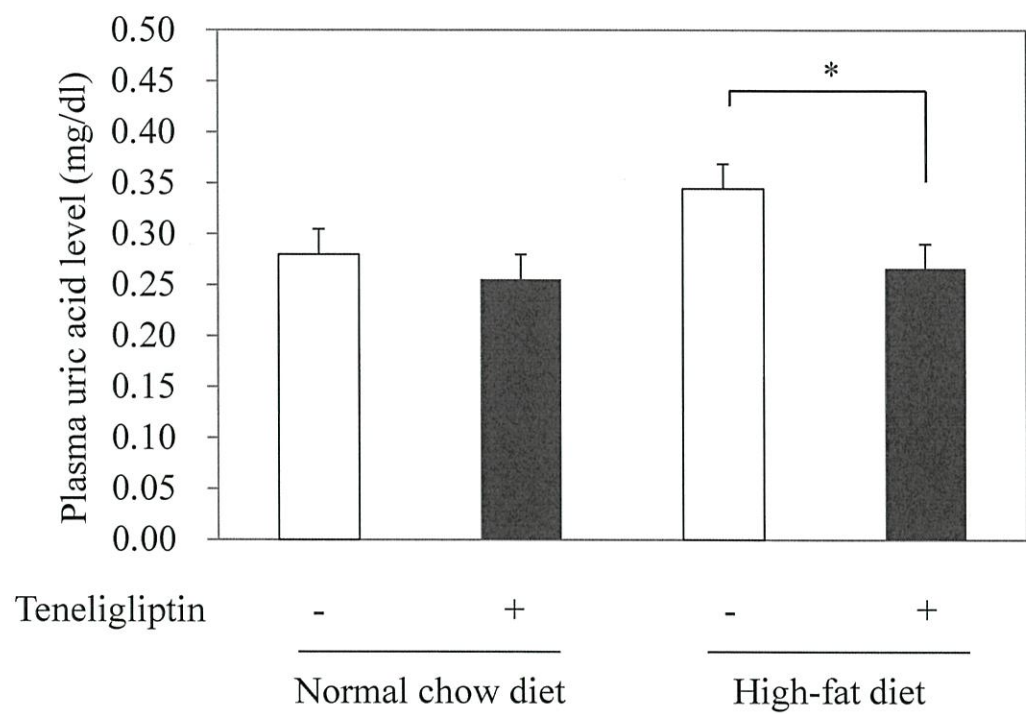
Figure 5. Effects of DPP-4 and teneligliptin on Xdh in 3T3-L1 adipocytes

Serum-starved 3T3-L1 adipocytes were treated without (open squares) or with DPP-4 (closed squares 200 ng/mL) and teneligliptin (1, 5, 10 μ M) for 12 hours. Total RNAs extracted from all cells were used for gene expression analysis of Xdh. Levels of Cph were used for normalization of sample loading. Data are the means \pm SEM of 3 independent experiments (1 experiment performed with 6 samples). Data are expressed relative to control values. *, $P < 0.05$; **, $P < 0.01$ vs DPP-4 treated control cells.

Figure 6. Schematic of the hypothetical mechanisms of improvement in uric acid metabolism by teneligliptin in adipose tissue

Teneligliptin decreases uric acid levels via the inhibition of DPP-4-induced Xdh expression in adipose tissue.

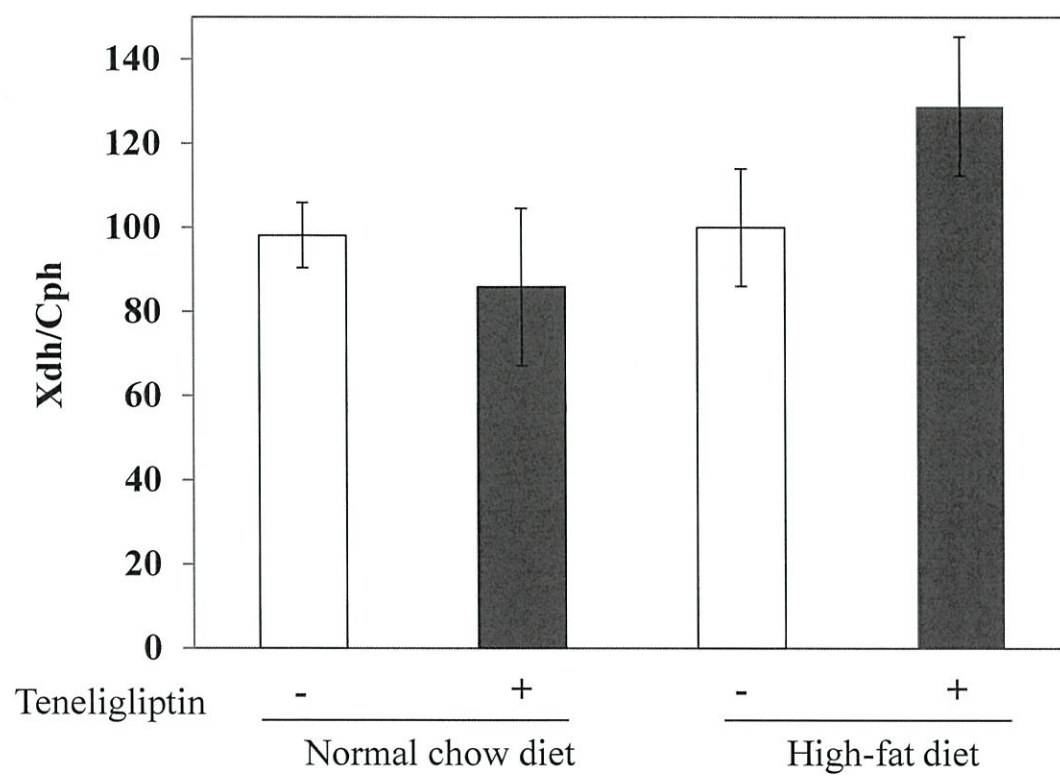
Figure 1



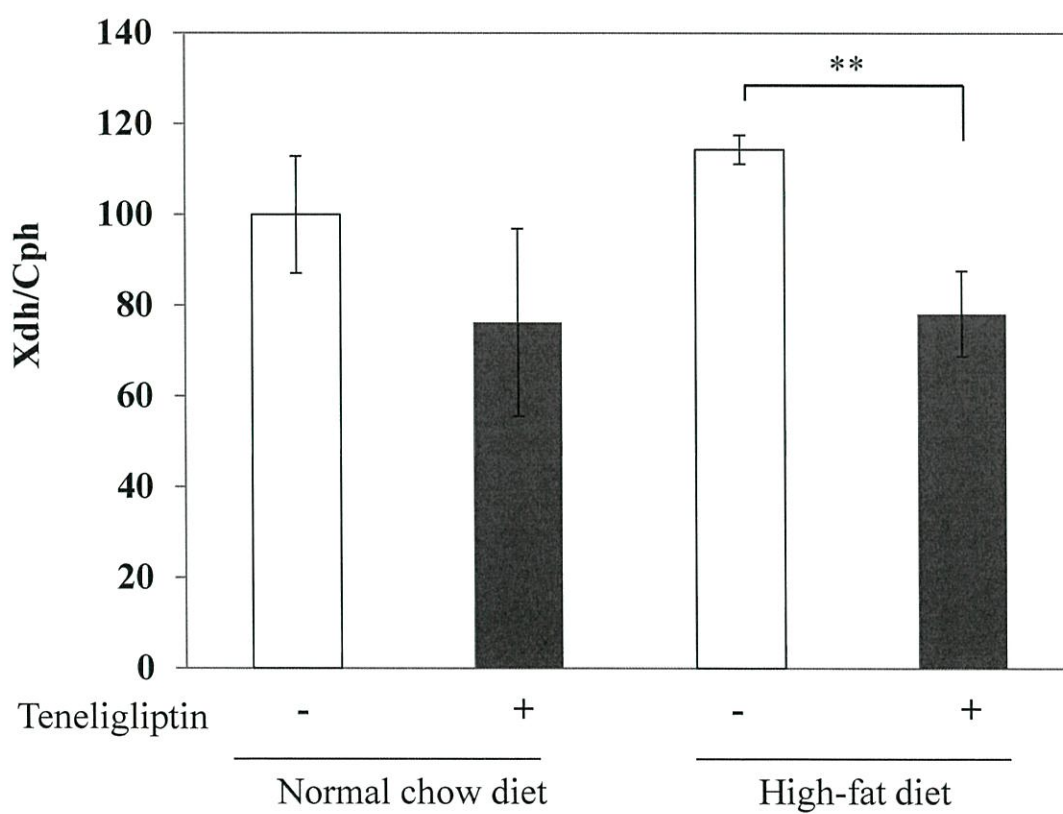
$*P < 0.05$

Figure 2

A. Liver tissue

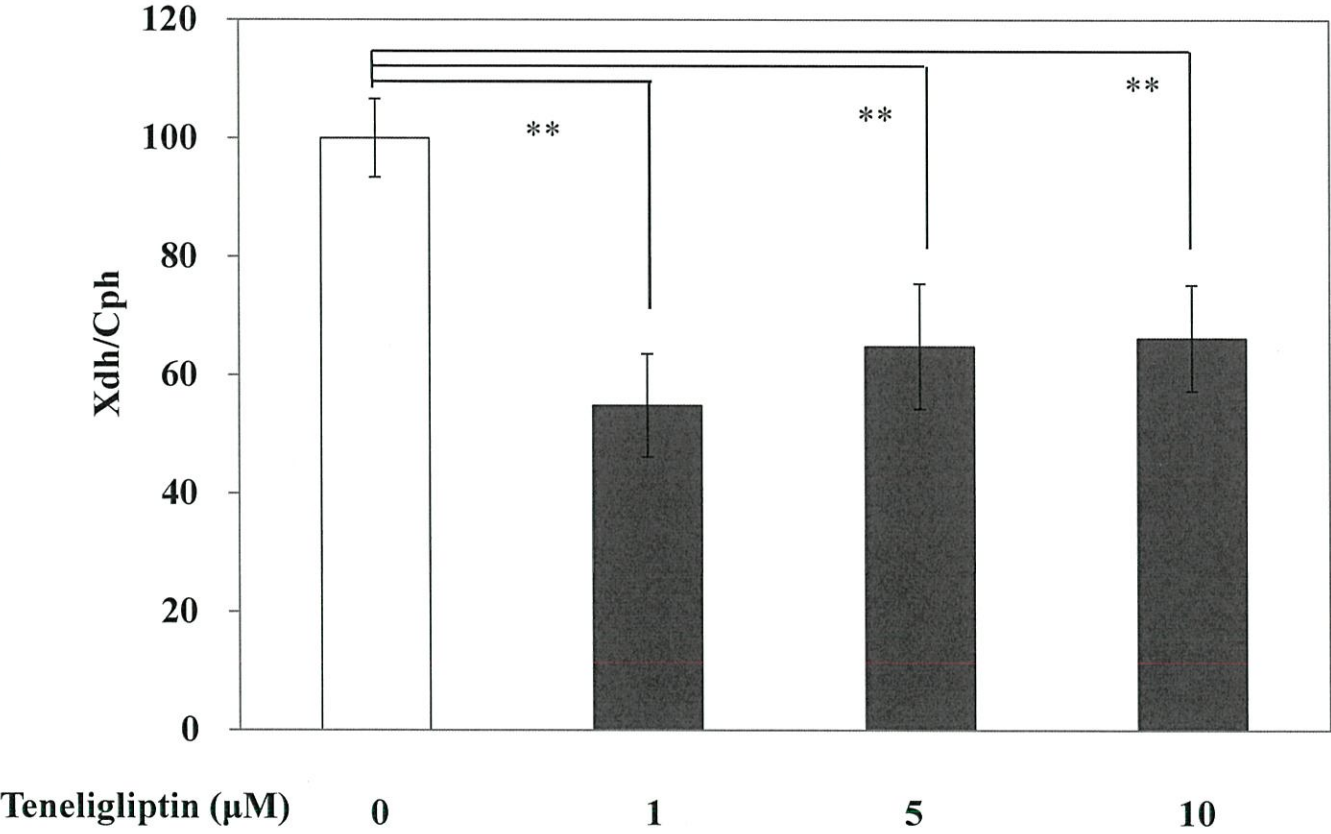


B. Epididymal adipose tissue



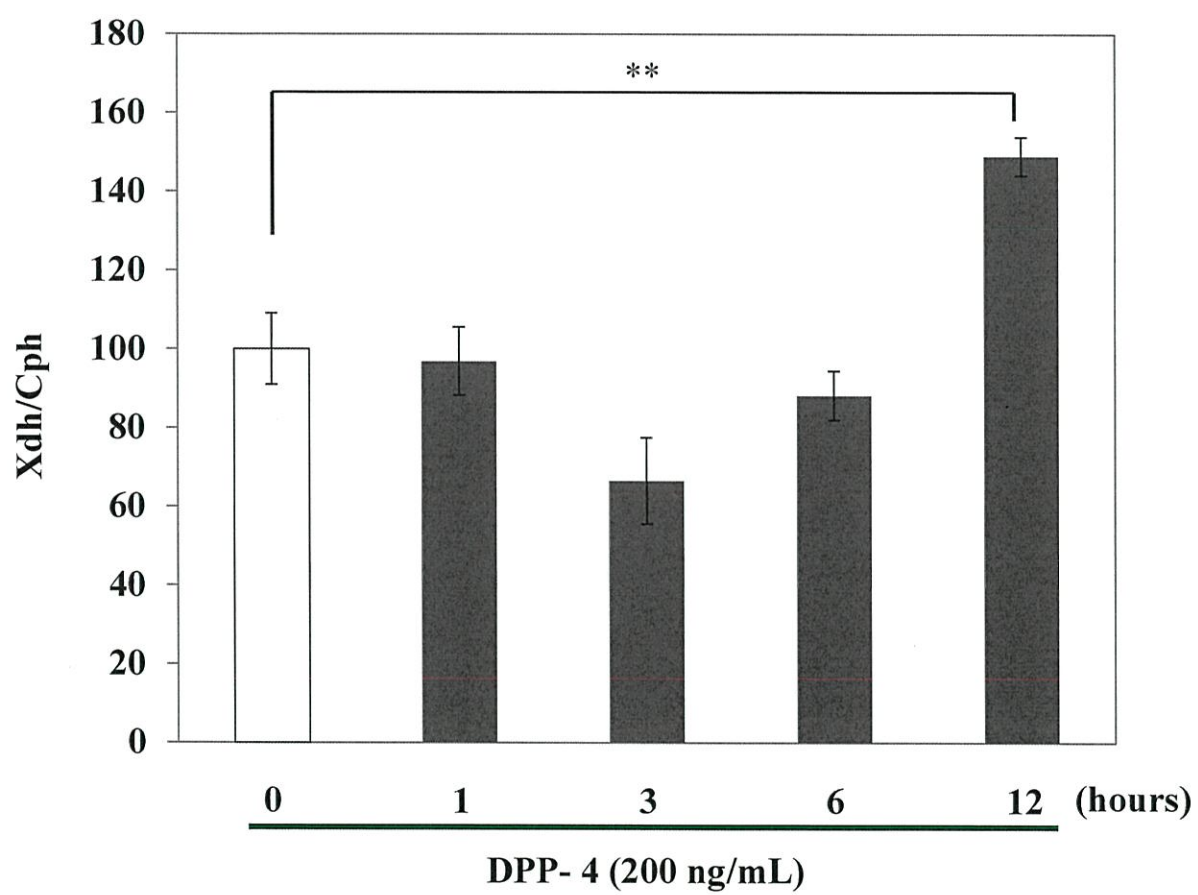
**** $P < 0.01$**

Figure 3



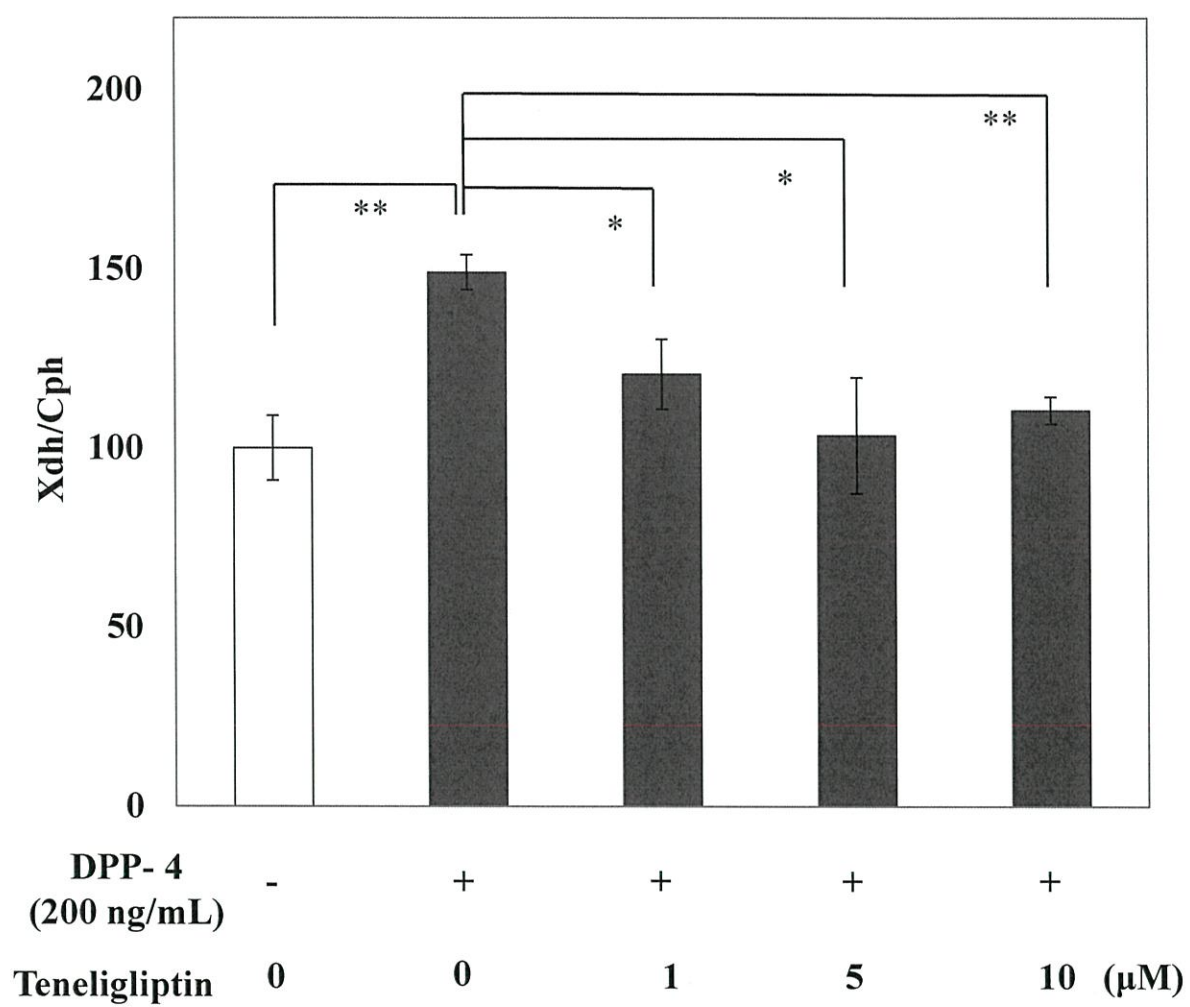
**** $P < 0.01$**

Figure 4



**** $P < 0.01$**

Figure 5



**** $P < 0.01$**

*** $P < 0.05$**

Figure 6

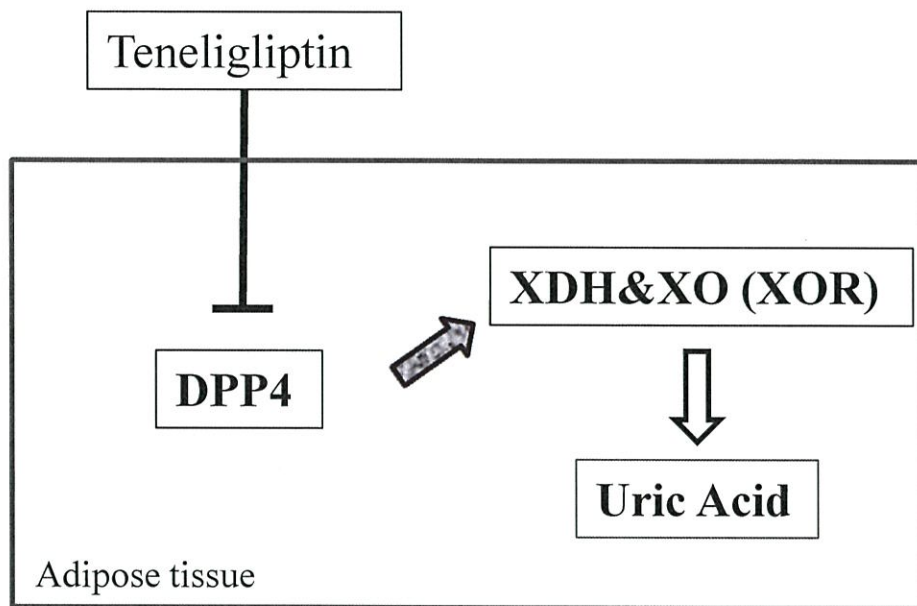


Table 1. Primers used for real-time RT-PCR

Target Sequence	Primers	Primer Sequence	Band size (bp)
Mouse Xdh MN_011723	mXdh-2819-F	5'- GGATGCTAATCGCAGAATAC -3'	120
	mXdh-2938-R	5'- GCTTCTGGTTGAAGTGAGTC -3'	
Rat Xdh NM_017154	rXdh-2579-F	5'- GAAGACTGGGACTGTAGTGG -3'	124
	rXdh-2702-R	5'- GGGGATCTTATAGGCGTTAT-3'	
Cyclophilin A (Cph) BC106030	Cph-96-F	5'- CTCCTTTGAGCTGTTTGCAG-3'	325
	Cph-420-R	5'-CACCACATGCTTGCCAT-3'	

Table 2. Body weight, food intake, and fasting plasma measurements in NCD-fed and HFD-fed rats

	Normal chow diet		High fat diet	
	Control	Teneligliptin	Control	Teneligliptin
Number	10	9	9	9
BW (g)	367.6 ± 6.9	355.8 ± 16.7	415.0 ± 10.3*	432.3 ± 9.3*
Food intake (g/day)	24.9 ± 0.6	24.4 ± 0.7	20.3 ± 0.3	20.6 ± 0.3
FPG (mg/dl)	158.0 ± 9.1	161.6 ± 9.1	163.0 ± 3.8	162.6 ± 2.8
Means ± SE				

Abbreviation: BW, body weight; FPG, fasting plasma glucose.

* $P < 0.05$ vs the normal chaw diet control rats